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## Ionophoric activity of the antibiotic peptaibol trichorzin PA VI: a $^{23}\text{Na}$ - and $^{35}\text{Cl}$ -NMR study

Delphine Duval <sup>a</sup>, Frank G. Riddell <sup>b</sup>, Sylvie Rebuffat <sup>a,\*</sup>, Nicole Platzer <sup>c</sup>,  
Bernard Bodo <sup>a</sup>

<sup>a</sup> Laboratoire de Chimie des Substances Naturelles, URA CNRS 401, GDR CNRS 790, Muséum National d'Histoire Naturelle,  
63 rue Buffon, 75231 Paris Cedex 05, France

<sup>b</sup> School of Chemistry, University of Saint Andrews, Saint Andrews, Fife KY16 9ST, UK

<sup>c</sup> Université Pierre et Marie Curie, Laboratoire de Chimie Structurale Organique et Biologique, 4 Place Jussieu, 75252 Paris Cedex 05,  
France

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### Abstract

Trichorzin PA VI (Ac Aib<sup>1</sup> Ser Ala Aib Iva Gln Aib Val Aib Gly<sup>10</sup> Leu Aib Pro Leu Aib Aib Gln Pheol<sup>18</sup>) is one of the seven main peptaibols forming the natural antibiotic 18-residue peptide mixture biosynthesised by a *Trichoderma harzianum* strain. Trichorzins exhibit antimycoplasmic activity resulting from membrane permeability perturbations. The membrane permeabilisation process by trichorzin PA VI has been examined in egg yolk phosphatidylcholine large unilamellar vesicles (LUV) and under conditions of ionic equilibrium by  $^{23}\text{Na}$ - and  $^{35}\text{Cl}$ -NMR experiments conducted in the presence of a chemical shift reagent and a relaxation agent, respectively. In such conditions, trichorzin PA VI exchanges both cations and anions across the vesicle bilayers, indicating the absence of ion- and charge-selectivity, in contrast to antibiotic ionophores, such as monensin or nigericin; the  $\text{Na}^+$  exchange is not influenced by the ionic strength. The kinetics of the  $\text{Na}^+$  exchange have been found to be third to fourth order with respect to the peptide concentration. The permeabilisation process of liposomes has been shown to be due to the formation of aggregates of three to four helical peptide monomers arranged into a supramolecular complex including presumably lipid molecules and forming a badly-defined pore in the bilayer. The major mechanism by which ions may exchange through the bilayer involves a long-lasting opening of the pores allowing complete exchange of the internal and external media in an 'all or nothing mode'. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Hydrophobic helix; Amphipathic helix; Ionophoric activity; Phospholipid bilayer;  $^{23}\text{Na}$ -NMR;  $^{35}\text{Cl}$ -NMR; Peptaibol; Trichorzin PA

### 1. Introduction

A series of seven antibiotic peptides, trichorzins PA, have been recently isolated from the soil fungus *Trichoderma harzianum* [1]. They are 18-residue linear peptides belonging to the peptaibol class which is exemplified by alamethicin [2]. Trichorzins PA are characterised by a high proportion of  $\alpha,\alpha$ -dialky-

Abbreviations: ePC, egg phosphatidylcholine; LUV, large unilamellar vesicles; OG, *n*-octyl- $\beta$ -D-glucopyranoside;  $\text{PPPi}$ , linear tripolyphosphate; SUV, small unilamellar vesicles; Aib,  $\alpha$ -aminoisobutyric acid; Iva, D-isovaline; Pheol, L-phenylalaninol

\* Corresponding author.

Fax: +33 (1) 4079-3147; E-mail: rebuffat@mnhn.fr

lated residues ( $\alpha$ -aminoisobutyric acid, Aib and isovaline, Iva), an acetylated N-terminus and a C-terminal amino alcohol, tryptophanol (Trp<sub>ol</sub>) or phenylalaninol (Phe<sub>ol</sub>); in addition, they differ between them by Aib for Iva substitutions at positions 4 and 7 (Fig. 1). The different trichorzins PA inhibit the growth of mycoplasma cells to the same extent; the antimycoplasmic activity has been shown to be related to their membrane perturbation properties [3]. These small wall-less bacteria are parasites of plants and animals including humans [4] and they are known to be pathogenic agents responsible for some serious diseases.

According to NMR and circular dichroism data, trichorzins PA are organised in amphipathic helices, mainly of the  $\alpha$ -type [1]. Fluorescence studies have shown that these peptides interact with the zwitterionic phospholipid bilayers of SUV and are embedded in the membrane, modifying its permeability; macroscopic conductance studies with planar bilayers have displayed a voltage- and concentration-dependence pattern [5]. The single-channel recordings exhibit a regular progression of conductance levels and especially the evolution of the open state probabilities with transmembrane potential argues for a typical alamethicin-like behaviour [6]. Similar ion currents have been shown to be promoted by alamethicins both in lipid vesicles (LUV) and planar bilayers, suggesting similar incorporation of the peptide in the two systems [7]. For such ionophoric peptides related to alamethicin, different models involving the formation of channels through the membrane bilayer are proposed to explain the ionophoric activity. The dynamic 'barrel-stave' model of channels of varying diameter, reflecting the monomer sequential uptake to or release from an aggregate of helical monomers, is the most commonly accepted [6,8]. In such a model, changes in the size of the pore are correlated to the multi-level conductance pattern. However, taking into account a study indicating that the size of the channel remains essentially unchanged, as the conductance levels increase [9], a second model was proposed by Cafiso [10]: in the 'multipore cluster' model, multistep conductances presumably result from the binding or dissociation of monomers or of non-conductive aggregates in the cluster. However, this last model appears questionable in the light of recent results [11], while the

barrel-stave model is supported once more by analysis of the conductance states of the channels formed by covalent dimers of alamethicin [12,13]. Whether one of these two models or some other proves to be correct, the circumstances involved in the transition from the non-conductive to the conductive state still remain speculative and several controversial hypotheses are proposed (for reviews, see [10,14]).

NMR studies have proved useful in the investigation of transport processes by ionophores, giving information on the kinetics and the selectivity for different ions. In particular, the mechanism of ion transport by the antibiotic ionophores, monensin, nigericin, M139603, narasin and salinomycin have been studied by heteronuclear NMR [15–18]. The kinetics observed have revealed that the transport mechanism of such ionophores involves the formation of a 1/1 complex between the ion and the ionophore, in agreement with the model proposed by Painter and Pressman [19]. The kinetics also allowed the determination of the relative ion selectivity. Similar kinetic studies performed on gramicidin A, a ionophoric peptide forming pores through the membranes by a non voltage-dependent mechanism, have shown that the association of two molecules was necessary to form the pore that allowed the ion efflux [20], a result which agreed with the widely accepted dimeric head to head model of ion channel [21].

In order to go further into the investigation of the mechanism giving rise to the ionophoric properties involved in the antimycoplasmic activity of trichorzins PA, we started a heteronuclear  $^{23}\text{Na}$ - and  $^{35}\text{Cl}$ -NMR study of the kinetics of the ion leakage induced in liposomes under conditions of ionic equilibrium, by trichorzin PA VI taken as a typical example of trichorzins PA. The anion/cation selectivity was examined. The formation of pores and the mechanism of ion transport involved in the biological properties are discussed by examination of the kinetics of the process including the reaction order with respect to the peptide.

## 2. Materials and methods

Trichorzin PA VI was isolated from the natural peptide mixture produced by *Trichoderma harzianum* (strain M-902608, Muséum National d'Histoire Na-

turelle, Paris) by a procedure making use of different chromatography steps and including HPLC (Kromasil, C18/5  $\mu\text{m}$  (AIT France);  $7.5 \times 300$  mm; MeOH/H<sub>2</sub>O 84/16; flow rate 2 ml/min; det. UV 220 nm;  $R_{t\text{PA VI}} = 106$  min). Purity of PA VI was shown to be 98% by analytical HPLC in the same system; its structure was determined by mass spectrometry and multidimensional NMR [1]. HPLC analyses were carried out with a Waters liquid chromatograph (6000A and M45 pumps, 680 automated solvent programmer, WISP 701 automatic injector, 481 UV-vis. detector). Egg yolk L- $\alpha$ -phosphatidylcholine type V-E (ePC) and *n*-octyl- $\beta$ -D-glucopyranoside (OG) were purchased from Sigma (St. Louis, MO) and used without further purification.

Egg PC large unilamellar vesicles (LUV) were prepared by dialytic removal of the detergent [22]. Typically, 69  $\mu\text{mol}$  ePC and 15 equivalents OG were dissolved in 3 ml of 100 mM (or 150 mM) NaCl solution. LUV of an average diameter of 600 nm were obtained by dialysis of this solution, 5 times for 12 h, against 2 l of 100 mM (or 150 mM) NaCl solution, at 40°C and were further used for  $^{23}\text{Na}$  experiments conducted with either 100 or 150 mM NaCl internally and externally the vesicles. For  $^{35}\text{Cl}$  studies, LUVs of an average diameter of 400 nm were prepared similarly, but dialysis was conducted at 4°C.

For the  $^{23}\text{Na}$ -NMR experiments, 4  $\mu\text{l}$  of 1 M  $\text{DyCl}_3$  were added to 1 ml of the above vesicle suspension and 1 ml of 100 mM choline chloride/20 mM  $\text{Na}_5\text{P}_3\text{O}_{10}$  to induce a chemical shift difference of 6.4 ppm between the  $^{23}\text{Na}_{\text{int}}^+$  and  $^{23}\text{Na}_{\text{out}}^+$  signals [15]. For the  $^{35}\text{Cl}$  experiments, 2  $\mu\text{l}$  of 1 M  $\text{MnBr}_2$  were added to 2 ml of vesicle suspension to induce a 200 times line broadening of the out  $^{35}\text{Cl}$  signal [23,24]. For

each peptide concentration obtained by adding 10–60  $\mu\text{l}$  of a 0.23 mM methanolic PA VI solution, 15–30 spectra were recorded as a function of time over a period of 1 h and the areas of the peaks were measured. The areas of the peaks due to internal  $^{23}\text{Na}$  (or  $^{35}\text{Cl}$ ) were expressed as the percentage of the total  $^{23}\text{Na}$  (or  $^{35}\text{Cl}$ ) concentration, with  $[\text{Na}^+]_{\text{int}} = \text{area Na}_{\text{int}}^+ / (\text{area Na}_{\text{int}}^+ + \text{area Na}_{\text{ext}}^+) \times 100$ . The  $^{23}\text{Na}$ -NMR spectra were obtained either on a Bruker AM 500 or MSL 500 or a Varian Unity 500 spectrometer, operating at 132.29 MHz and equipped with a 10-mm probe. Each spectrum was acquired with: spectral width 3000 Hz, 1 K data points, 2000 free induction decays from 90° pulses and a 0.2 s relaxation delay. The  $^{35}\text{Cl}$  NMR spectra were performed on a Bruker MSL 500 spectrometer operating at 49.00 MHz; spectral width 10000 Hz, 8 K data points, 20000 free induction decays from 90° pulses, relaxation delay 0.1 s. The field-frequency ratio was locked on the  $^2\text{H}$  resonance of  $\text{D}_2\text{O}$  contained in the inner compartment of a 4 mm insert; all spectra were recorded at 303 K.

### 3. Results

#### 3.1. Characterisation of the ionophoric activity of trichorzin PA VI

The ionophoric activity of trichorzin PA VI in the transport of monovalent cations through PC bilayers, was studied by heteronuclear  $^{23}\text{Na}$ -NMR. Suspensions of large unilamellar vesicles (LUV) were prepared with 100 mM NaCl inside and outside the vesicles. The  $^{23}\text{Na}$ -NMR spectrum was followed over a period of 1 h, after the addition of  $\text{DyCl}_3$ /

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
<b>PA VI</b>	Ac	Aib	Ser	Ala	<b>Aib</b>	Iva	Gln	<b>Aib</b>	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	<b>Pheol</b>
<b>PA II</b>	Ac	Aib	Ser	Ala	<b>Aib</b>	Iva	Gln	<b>Aib</b>	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	<b>Trpol</b>
<b>PA IV</b>	Ac	Aib	Ser	Ala	<b>Aib</b>	Iva	Gln	<b>Iva</b>	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	<b>Trpol</b>
<b>PA V</b>	Ac	Aib	Ser	Ala	<b>Iva</b>	Iva	Gln	<b>Aib</b>	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	<b>Trpol</b>
<b>PA VII</b>	Ac	Aib	Ser	Ala	<b>Iva</b>	Iva	Gln	<b>Iva</b>	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	<b>Trpol</b>
<b>PA VIII</b>	Ac	Aib	Ser	Ala	<b>Aib</b>	Iva	Gln	<b>Iva</b>	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	<b>Pheol</b>
<b>PA IX</b>	Ac	Aib	Ser	Ala	<b>Iva</b>	Iva	Gln	<b>Aib</b>	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	<b>Pheol</b>

Fig. 1. Sequences of trichorzin PA VI and of the other isolated trichorzins PA forming the natural antibiotic peptide mixture from *T. harzianum* [1].

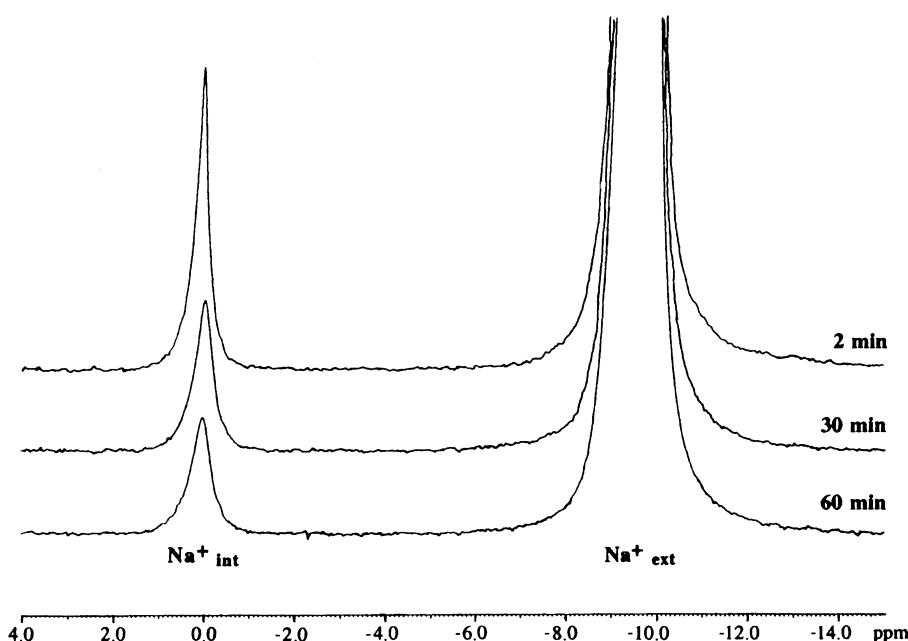


Fig. 2. A time series of  $^{23}\text{Na}$ -NMR spectra (132.29 MHz, 303 K) of LUV containing 100 mM NaCl, showing the decrease of  $\text{Na}^+_{\text{int}}$  on addition of 2.3  $\mu\text{M}$  PA VI (3 mM  $\text{DyCl}_3$  allowing to create the  $\text{DyCl}_3/\text{Na}_5\text{P}_3\text{O}_{10}$  shift reagent and to generate a shift difference of approximately 9.8 ppm). The  $[\text{Na}^+]_{\text{int}}$  was determined as indicated in Section 2.

$\text{Na}_5\text{P}_3\text{O}_{10}$  as chemical shift reagent [15] which allowed distinction between intra- ( $\text{Na}^+_{\text{int}}$ ) and extravesicular ( $\text{Na}^+_{\text{ext}}$ ) ions by inducing an upfield shift of the extravesicular  $\text{Na}^+$  ions. The addition of small aliquots of trichorzin PA VI to the vesicular suspension leading to [peptide]/[lipid] molar ratios in the range  $10^{-4}$  to  $7 \times 10^{-4}$ , resulted in a decrease in the area of the peak due to the non-shifted  $\text{Na}^+$  ions ( $\text{Na}^+_{\text{int}}$ ) as a function of time (Fig. 2). The trichorzin PA VI ionophoric activity toward  $\text{Na}^+$  cations was thus characterised.

Furthermore, adding a supplementary aliquot of chemical shift reagent after observation of the ion transport induced by trichorzin PA VI resulted in three populations of  $\text{Na}^+$  ions (Fig. 3). An upfield shift of the extravesicular  $\text{Na}^+_{\text{ext}}$  ions was noticed ( $\text{Na}^+_{\text{ext}} + \text{DyCl}_3$  [2]), together with the presence of a weak residual signal at the previous  $\text{Na}^+_{\text{ext}}$  chemical shift ( $\text{Na}^+_{\text{int}} + \text{DyCl}_3$  [1]), which was assigned to vesicles loaded with both  $\text{Na}^+$  ions and chemical shift reagent. Finally, the signal at the initial unshifted position of internal  $\text{Na}^+$  ( $\text{Na}^+_{\text{int}}$ ) resulted from unperturbed vesicles. It is worth noting the loss of around 25% of the total integrated  $^{23}\text{Na}_{\text{int}}$  signals.

The decrease in the internal non-shifted signal in-

tensity (Fig. 2) did not appear to be due to vesicle lysis, since measurement of the scattering peak intensity during the ion transport process (not shown) did not show any significant change, suggesting that the size of the liposomes was not modified.

### 3.2. Orders of the exchange kinetic

The absence of linear relationship between  $[\text{Na}^+]_{\text{int}}$  and the time indicated that, as expected, trichorzin PA VI did not transport ions by the mechanism involving a 1/1 ion/ionophore complex, which has been shown previously for nigericin and salinomycin [17,18]. This was confirmed by the linear relationship between  $1/[\text{Na}^+]_{\text{int}}$  and the time, observed for each peptide concentration (Fig. 4A).

The general rate law for the ion transport from the interior of the vesicle can be expressed by:

$$-\frac{d[\text{Na}^+]_{\text{int}}}{dt} = k[\text{Na}^+]_{\text{int}}^n \left( \frac{[\text{peptide}]}{[\text{lipid}]} \right)^m \quad (1)$$

where  $[\text{Na}^+]_{\text{int}}$  is the mole fraction of  $\text{Na}^+$  ions inside the vesicles as regards the total  $[\text{Na}^+]$ ,  $n$  is the order of the transport with respect to the mole fraction of the total sodium inside the vesicles, and  $m$  is the order of the transport with respect to the concentra-

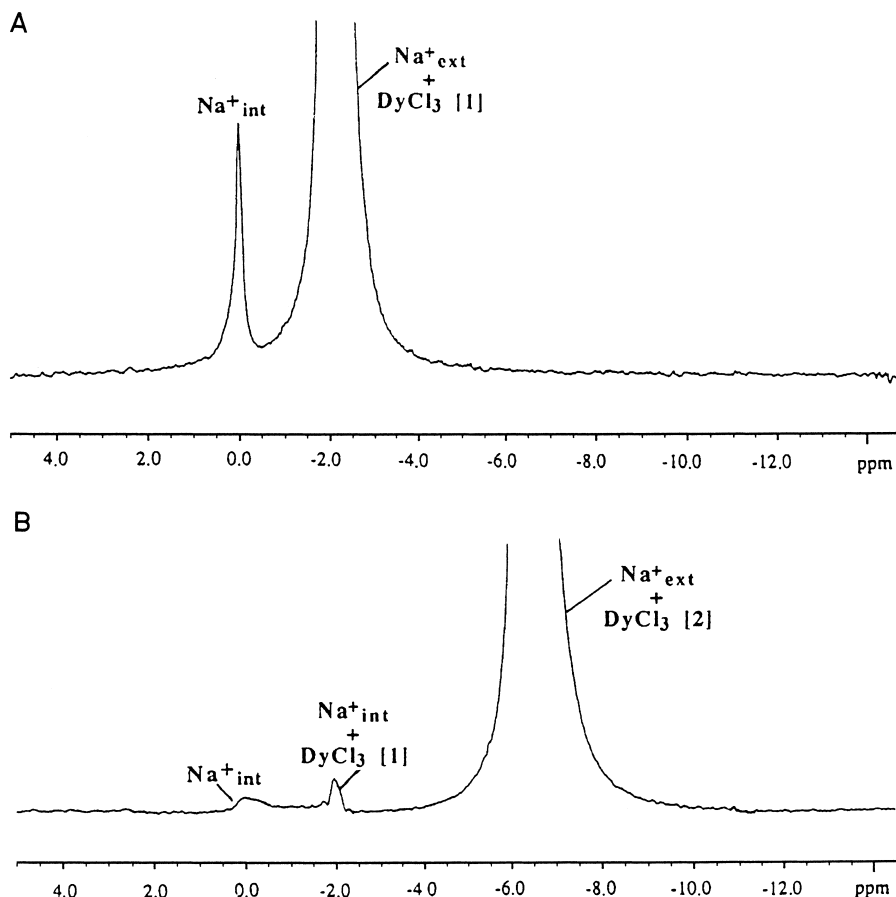


Fig. 3. Observed changes in  $^{23}\text{Na}$ -NMR spectra (132.29 MHz, 303 K) of LUV containing 100 mM NaCl, on addition of 2.3  $\mu\text{M}$  PA VI and (A) 0.8 mM  $\text{DyCl}_3$  to generate a shift difference of approximately 2.4 ppm. (B) 2 mM  $\text{DyCl}_3$  to generate a shift difference of approximately 6.4 ppm.

tion of peptaibol in the membrane expressed as the  $[\text{peptide}]/[\text{lipid}]$  molar ratio and assuming that all the added peptaibol is associated to the bilayer.

The order of the transport with respect to the peptaibol concentration could thus be determined according to two different methods. The first one consisted in plotting  $1/[\text{Na}^+]_{\text{int}}$  as a function of time; the resulting straight line allowed measurement of the slope  $K$  which could be expressed by:

$$K = k \left( \frac{[\text{peptide}]}{[\text{lipid}]} \right)^m \quad (2)$$

Thus, the plot of  $\ln K$  as a function of the peptide concentration resulted in a straight line, the slope of which being equal to  $m$ , the order of the kinetic (Fig. 4B). By this method, the order was found to be  $3.4 \pm 0.5$ .

The second way to determine  $m$  involved the meas-

urement of the initial rates ( $v$ ) of the kinetics obtained for different peptide concentrations. The tangents to the plots of  $[\text{Na}^+]_{\text{int}}$  as a function of time gave a measure of these rates (Fig. 5A). The linear relationship obtained between  $\ln(v)$  and  $\ln([\text{pep}]/[\text{lip}])$  (Fig. 5B) gave the slope  $m$  which was found to be  $3.1 \pm 0.5$ . The exchange kinetics were thus found third or fourth order with respect to the peptide concentration. Finally, comparison of the above parameters with those measured for 150 mM NaCl vesicles showed the absence of influence of the ionic strength on the kinetics.

### 3.3. Ion selectivity

In order to probe the ion selectivity for anions or cations and to check that  $\text{Cl}^-$  ion exchange was occurring, a  $^{35}\text{Cl}$ -NMR study using  $\text{Mn}^{2+}$  as relaxation

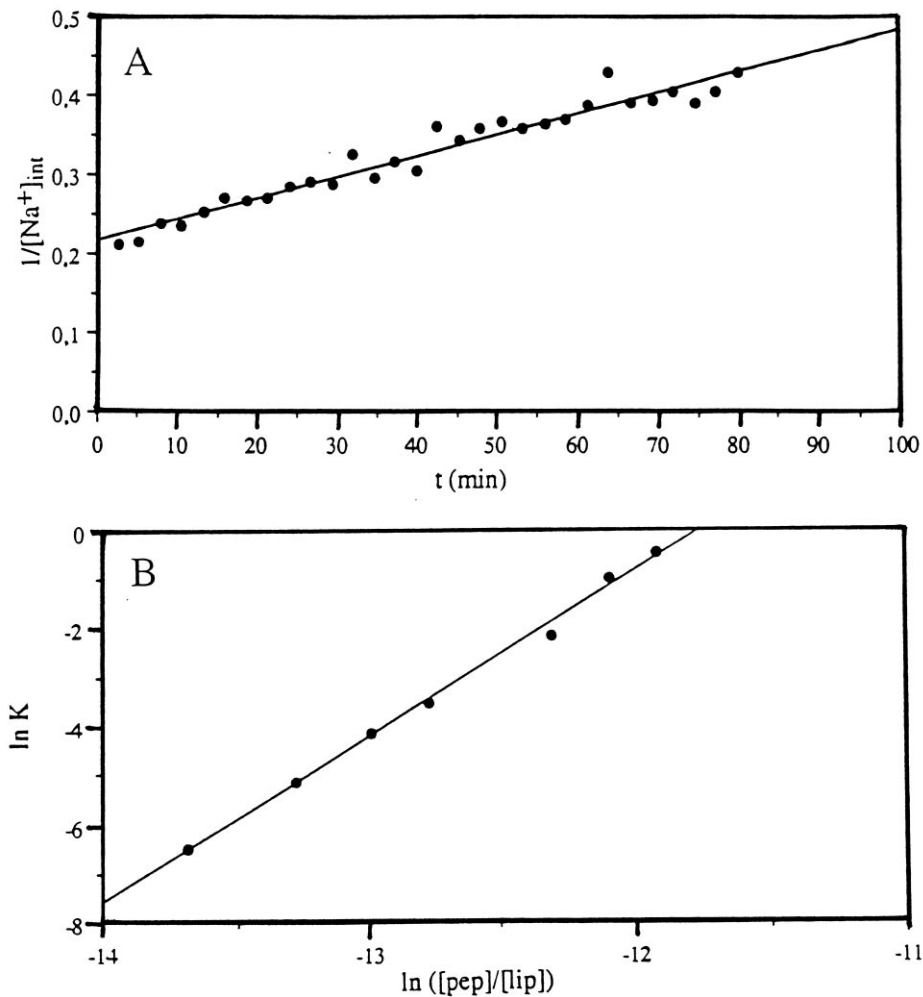


Fig. 4. (A) Graph of  $1/[\text{Na}^+]_{\text{int}}$  versus time (min) allowing determination of the slope  $K$  (ePC LUV, 100 mM NaCl, 3.4  $\mu\text{M}$  trichorzin PA VI). (B) Graph of  $\ln K$  versus  $\ln [\text{pep}]/[\text{lip}]$  allowing determination of the order of the kinetic with respect to the peptide concentration.

agent [23,24] was undertaken. Smaller LUV of an average diameter of 400 nm were used, as  $\text{Mn}^{2+}$  ions promote bursting and fusion of the larger LUVs [23]. However, the smaller size of the vesicles and the lower sensitivity of  $^{35}\text{Cl}$ -NMR compared to  $^{23}\text{Na}$ -NMR resulted in lower signal to noise ratio and greater difficulty in the quantification of the  $\text{Cl}_{\text{int}}^-$  signal, due to the superimposition of the relatively sharp  $\text{Cl}_{\text{int}}^-$  signal with the very broad  $\text{Cl}_{\text{ext}}^-$  signal, only allowing a qualitative study. However, the results were consistent with the  $\text{Cl}_{\text{int}}^-$  signal decreasing at a similar rate to that of  $^{23}\text{Na}^+$  and showed the leakage of  $\text{Cl}^-$  outside the vesicles and entrance of  $\text{Mn}^{2+}$  into the vesicles. This indicated the

absence of ion selectivity, as addition of trichorzin PA VI to the vesicles exchanged cations as well as anions.

#### 4. Discussion

Preliminary experiments, using the time-course measurement method described by Pike et al., which implies replacement of the  $\text{Na}_{\text{ext}}^+$  of the vesicle suspension by  $\text{Li}^+$  [25], have shown a fast efflux of  $\text{Na}^+$  ions upon the peptaibol addition, in the absence of a chemical potential. However, the area of the  $\text{Na}_{\text{ext}}^+$  resonance signal rapidly increased at the expense of

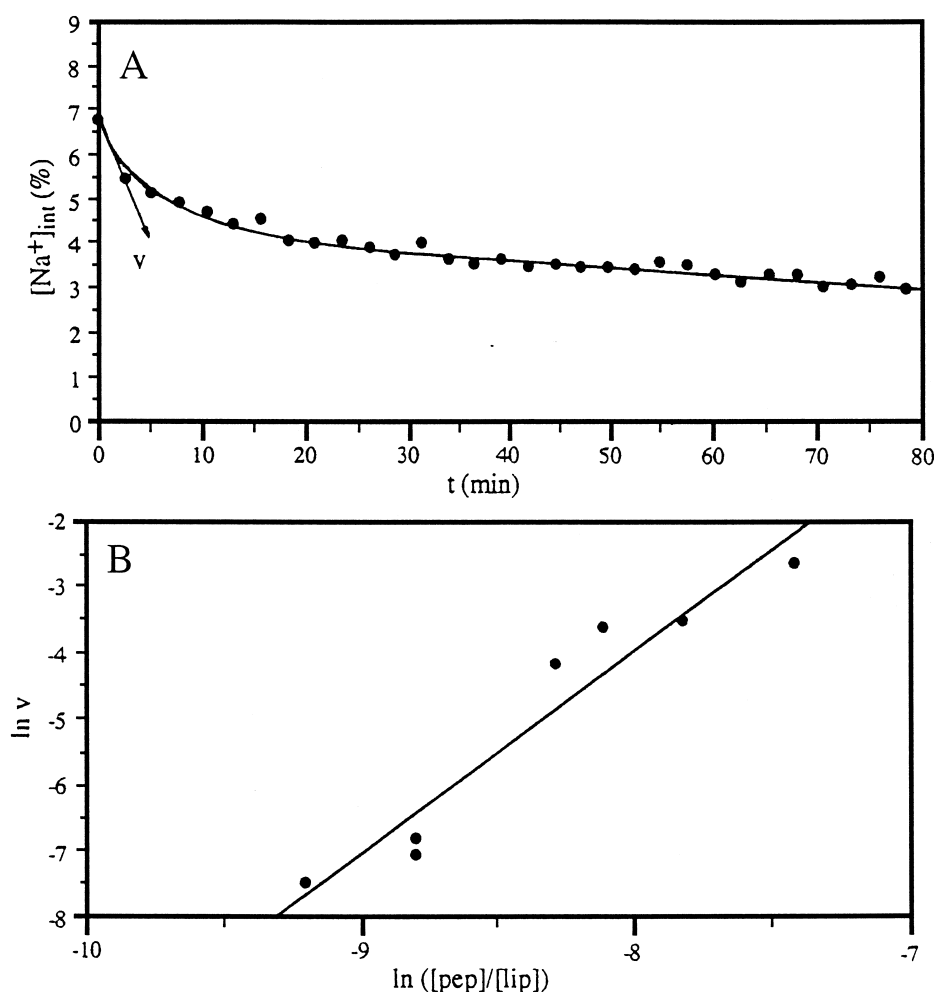


Fig. 5. (A) Graph of  $[\text{Na}^+]_{\text{int}}$  versus time (min) allowing determination of the initial rate  $v$  (ePC LUV, 100 mM NaCl, 2.9  $\mu\text{M}$  of trichorzin PA VI). (B) Graph of  $\ln v$  versus  $\ln ([\text{pep}]/[\text{lip}])$  allowing determination of the order of the kinetic with respect to the peptide concentration.

the  $\text{Na}^+_{\text{int}}$  signal for about 5–10 minutes after peptide addition, preventing accurate monitoring of the process.

We thus decided to examine the ionophoric properties of peptaibols by using the  $^{23}\text{Na}$ -NMR method under conditions of ionic equilibrium developed by one of us [15–18]. We were initially looking for line broadening similar to that shown by the ionophoric antibiotics, but instead found that the integrated intensity of the  $\text{Na}^+_{\text{int}}$  decreased as a function of time.

Furthermore, the transport rate was shown to be third or fourth order with respect to the trichorzin PA concentration (Figs. 4 and 5), indicating the necessity of association of three to four peptide monomers to allow ion efflux. The number of monomers

assumed to make up the pores or channels formed by peptaibols have been evaluated previously, both in vesicle and in planar bilayer systems. Four monomers have been shown to compose the conducting aggregate formed in LUV by alamethicin [7], while the number of monomers determined for alamethicin and analogues in planar bilayers ranges between 3 and 11, depending on the experimental conditions, including hydration and bilayer composition, and on the number and nature of residues in the peptide [14,26–29]. The formation of pores made up of peptide aggregates which involve three to four peptide monomers is thus suggested, consistent with the barrel-stave model of the peptaibol ion channels which involves the aggregation of transbilayer helical pep-

tide monomers. However, no consensus seems to appear in the literature about the definition of the pores, as this term is sometimes used to describe a channel of a well-defined structure and sometimes means a peptide-involved structural defect in the membrane through which ions can pass. The latter broader definition is used in the following, but without discarding the possible occurrence of transbilayer helices arranged in a parallel fashion to form a well-defined channel. Moreover, our experiments are performed under equilibrium conditions, while the voltage-gated channel activity is measured in the presence of a transbilayer voltage and thus under profoundly non-equilibrium conditions. Thus, it is likely that the ion permeability in the current study does not refer to the same molecular entity as the voltage-gated channel experiments.

The NMR results obtained in LUV and in the absence of potential allow us to draw further conclusions concerning the structure and lifetime of the pores involved in the membrane permeabilisation process by trichorzins PA under equilibrium conditions. The results tell us that the internal unshifted  $\text{Na}^+$  pool is decreasing with time. Two distinct extreme types of exchange of the internal and external contents of the vesicles can be envisaged to account for this. In the first one, the 'gradual mode', a pore opens for a small period of time, allowing partial exchange between the internal and external media. In the other one, the pore opens for a longer period of time, allowing complete exchange of the internal and external media. We call the second type of exchange the 'all or nothing mode'. Between these two extremes, there will be a spectrum of possibilities depending on the lifetime of the pore. These two distinct extreme processes have been distinguished herein by studying the  $^{23}\text{Na}$  spectrum obtained upon addition of a supplementary aliquot of chemical shift reagent, after a first exchange experiment (Fig. 3). An upfield shift of the  $\text{Na}_{\text{ext}}^+$  signal was observed concomitantly with the persistence of a weak signal at the previous  $\text{Na}_{\text{ext}}^+$  chemical shift. The observation of three populations of  $\text{Na}^+$  ions (Fig. 3), which could be assigned to  $\text{Na}_{\text{int}}^+$ , ( $\text{Na}_{\text{int}}^+ + 0.8 \text{ mM}$  of  $\text{DyCl}_3/\text{Na}_5\text{P}_3\text{O}_{10}$ ) and ( $\text{Na}_{\text{ext}}^+ + \text{additional } 1.2 \text{ mM}$  of  $\text{DyCl}_3/\text{Na}_5\text{P}_3\text{O}_{10}$ ) may be interpreted by the presence of intact vesicles loaded with both  $\text{Na}^+$  ions and chemical shift reagent, con-

sistent with a total exchange of the inside and outside media. On the other hand, a partial exchange, as observed in the 'gradual mode' should lead to a series of  $\text{Na}^+$  frequencies, gradually shifted toward higher field with respect to the initial internal  $\text{Na}^+$  signal; the intensity of the sodium signals would thus be smeared out, due to the random distribution of the shift reagent. This was not observed, suggesting that we are in the presence of long-lived pores and that the mechanism lies towards the 'all or nothing' end of the spectrum of possibilities. A mechanism involving mainly permanent bursting of vesicles appears to be ruled out, both by the light scattering experiments that do not show any change in the particle size range with time and by the  $^{23}\text{Na}$ -NMR experiments. However, a slight contribution of this last mechanism can be envisaged, as the loss of 25% of the integrated  $^{23}\text{Na}_{\text{int}}^+$  signals was observed. The  $\text{Na}^+$  exchange process induced by trichorzin PA VI would thus mainly involve the formation of long-life pores in the bilayer, allowing complete exchange of the internal and external media. Although the involvement of another type of process could not be completely rejected according to the sensitivity of the technique, most of the exchange process has to involve the 'all or nothing' mode: there will presumably be a Gaussian distribution of times for which the pores are open and our experiment shows that the average time for which a pore is open is at, or close to, the time required for complete exchange of vesicle content. Furthermore, the pores allow entrance of the chemical shift reagent inside the vesicles. Strictly speaking, the  $^{23}\text{Na}^+$  results described herein arise from exchange of the  $\text{Dy}(\text{PPP}_i)_2^{7-}$  shift reagent through the pores and exchange of sodium ions must be at least as rapid as the diffusion of the much larger anionic shift reagent. The fact that the shift reagent enters into the vesicles also reveals the absence of selectivity of the pores, in agreement with the fact that both  $\text{Na}^+$  and  $\text{Cl}^-$  ions exchange through the bilayer. The lack of selectivity supports a mechanism involving pores made up of peptide aggregates into the membrane.

## 5. Conclusion

Therefore, the formation of trimeric or tetrameric



aggregates of peptaibol helices to form a pore in the membrane with a long-lasting opening to allow complete exchange of internal and external ions appears to be involved in the ion exchange process by trichorzin PA VI in liposomes. Taking into account that the size of the pore has to be large enough to allow entrance of the chemical shift reagent inside the liposomes, the picture that emerges from the data is that of a supramolecular complex including an aggregate of three to four helical peptide monomers together with lipid molecules, making up a badly-defined pore. The distinction between the 'gradual' and 'all or nothing' mechanisms has been done previously for melittin [30], mastoparan [31] and tachyplesin [32], by monitoring the apparent efflux of a fluorescent marker. The present study shows that the main process by which ions can exchange through the pores involves the 'all or nothing' mode. This is the first time that such a distinction is made for peptaibols and that  $^{23}\text{Na}$ - and  $^{35}\text{Cl}$ -NMR have been used for this purpose. This study allows the proposal of a mechanism for the structure and function of the pores formed in liposomes in the absence of potential and under equilibrium conditions by the peptaibol trichorzin PA VI. NMR analysis of ion exchange through liposome bilayers is confirmed as a suitable method for the study of the mechanism by which membranotropic compounds can permeabilise membrane bilayers.

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